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# Exploring the interactions of interferon-gamma and polyphenols in colorectal cancer cells

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## Introduction

The immune system possesses the ability to recognize and regulate the development of tumours. Adaptive Immune responses play a central in tumour immune surveillance, in particular T-cells secreting interferon-gamma (IFN-γ). However, tumour cells can breach the host's immune system in a multitude of ways: IFN-γ resistance, reduction of antigen expression, loss of MHC's and /or the production of immuno-inhibitory ligands (Dunn *et al* 2004). Cancers can utilize the expression of cell surface ligands to evade the immune system. Here we investigate the effect of IFN-γ and polyphenols on Programmed Cell Death-Ligand 1 (PD-L1), which is believed to help cancers cell evade the immune system. PD-L1 is a ligand that has been shown to inhibit immune responses via the Programmed Cell Death-1 receptor on T-cells (Figure 1) (Juneja *et al* 2017). We are also interested to observe the effects of IFN-γ and polyphenols on other cellular processes such as cell cycle and apoptosis.

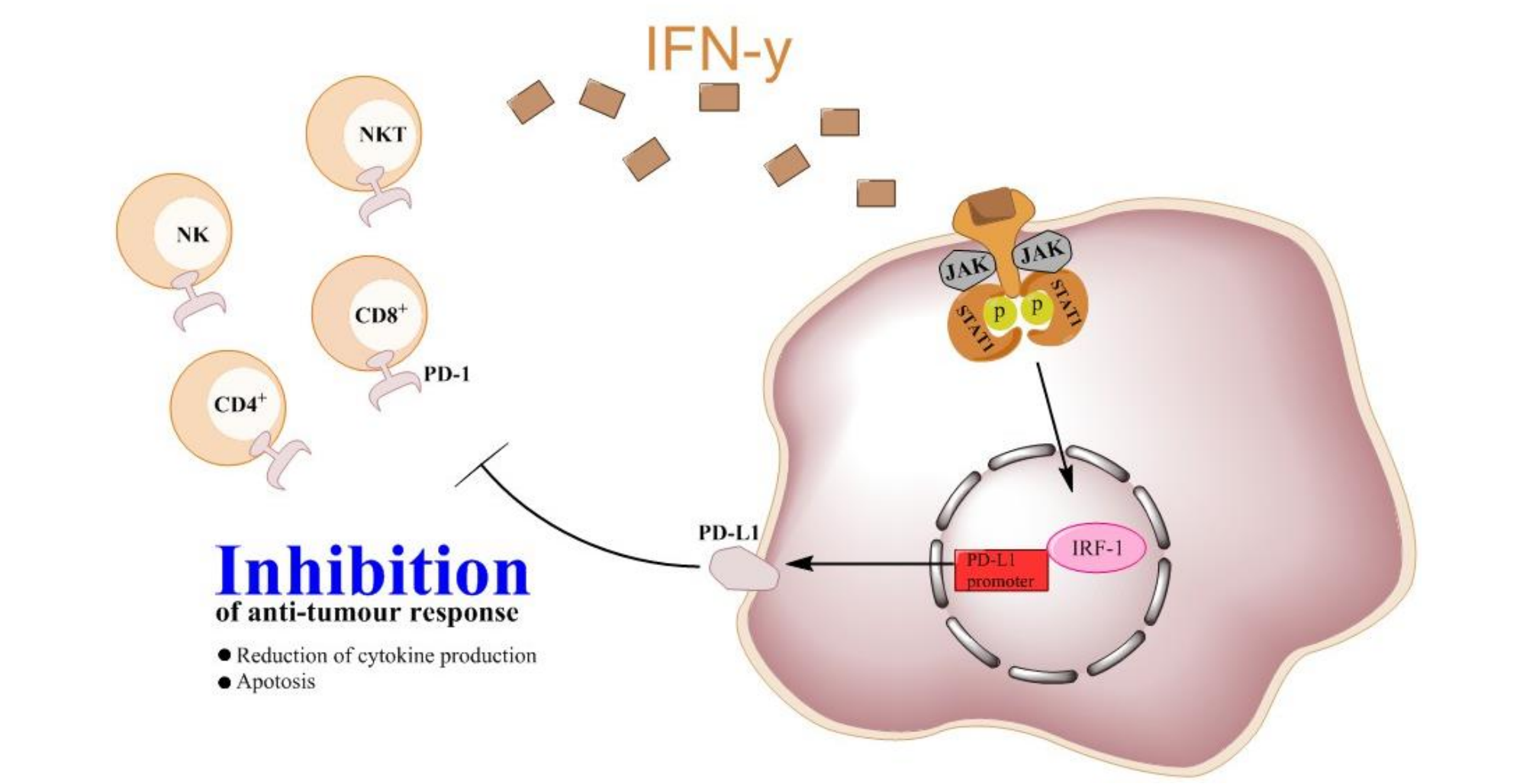


Figure 1: A simplified signally pathway of the induction of PD-L1 by IFN-γ and the subsequent immuno-inhibitory response.

## Methods

**Cell culture** - Patient matched Primary adenocarcinoma SW480 and metastatic SW620 human colorectal cancer (CRC) cell lines were cultured in DMEM supplemented with 10% (v/v) fetal calf serum and 100 µg/ml Penicillin-streptomycin and maintained at 37°C with 5% CO<sub>2</sub>.

**Cell surface staining** - PD-L1 was fluorescently stained using anti-human PD-L1 APC antibodies and assessed by flow cytometry (BD FACs Calibur) data was analyzed using FlowJo software.

**Cell cycle analysis** - Cell cycle analyses were performed on both colorectal cell lines using propidium iodide staining and flow cytometry. Data was analyzed using the Deans-Jetts-Fox model and manually set gates.

**Cell titre glo assay** - Cells were incubated with 20ng/ml of IFN-γ, 0-500 µM of apigenin and 0-500 µM of quercetin for 24 hrs. Treatment data was normalized to the 0.1% ethanol vehicle controls which was assigned 100% cell viability.

**Statistical assessment** - Normality of all data sets was statistically analysed by a Shapiro-Wilk test. Statistical assessment was made using a Kruskal-Wallis with a Conover Inman post hoc test. Results were considered statistically significant when  $P < 0.05$ .

## Results

### Interferon-gamma induces PD-L1 in SW480 & SW620 CRC cell lines

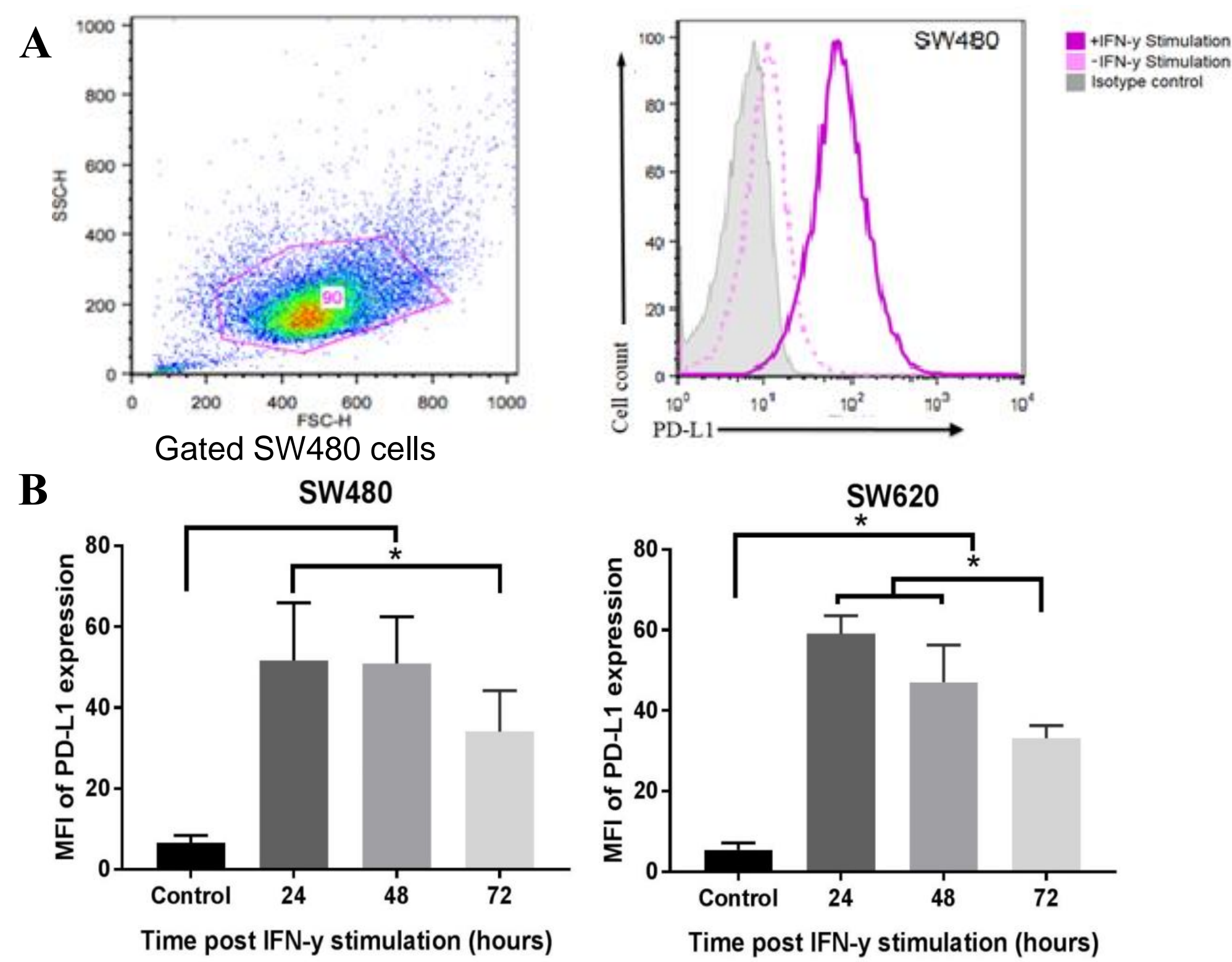


Figure 2: (A) Representation of gated SW480 cells and PD-L1 expression histograms. (B) Both SW480 and SW620 cells were incubated with 20 ng/ml of IFN-γ for 24, 48 and 72 hours, and the median fluorescent intensity (MFI) were compared against the MFI of unstimulated cells (Control). Treatments were run in triplicate in two independent experiments; data is expressed as mean ± standard deviation. Statistical significance was set at  $P < 0.05$  and is denoted <sup>\*</sup>.

IFN-γ induced a significant upregulation of PD-L1 expression on the primary and metastatic colorectal cell lines after 24 hrs compared to unstimulated controls (Figure 2).

### Preliminary data of the effect of interferon-gamma primary and metastatic CRC cell cycle progression

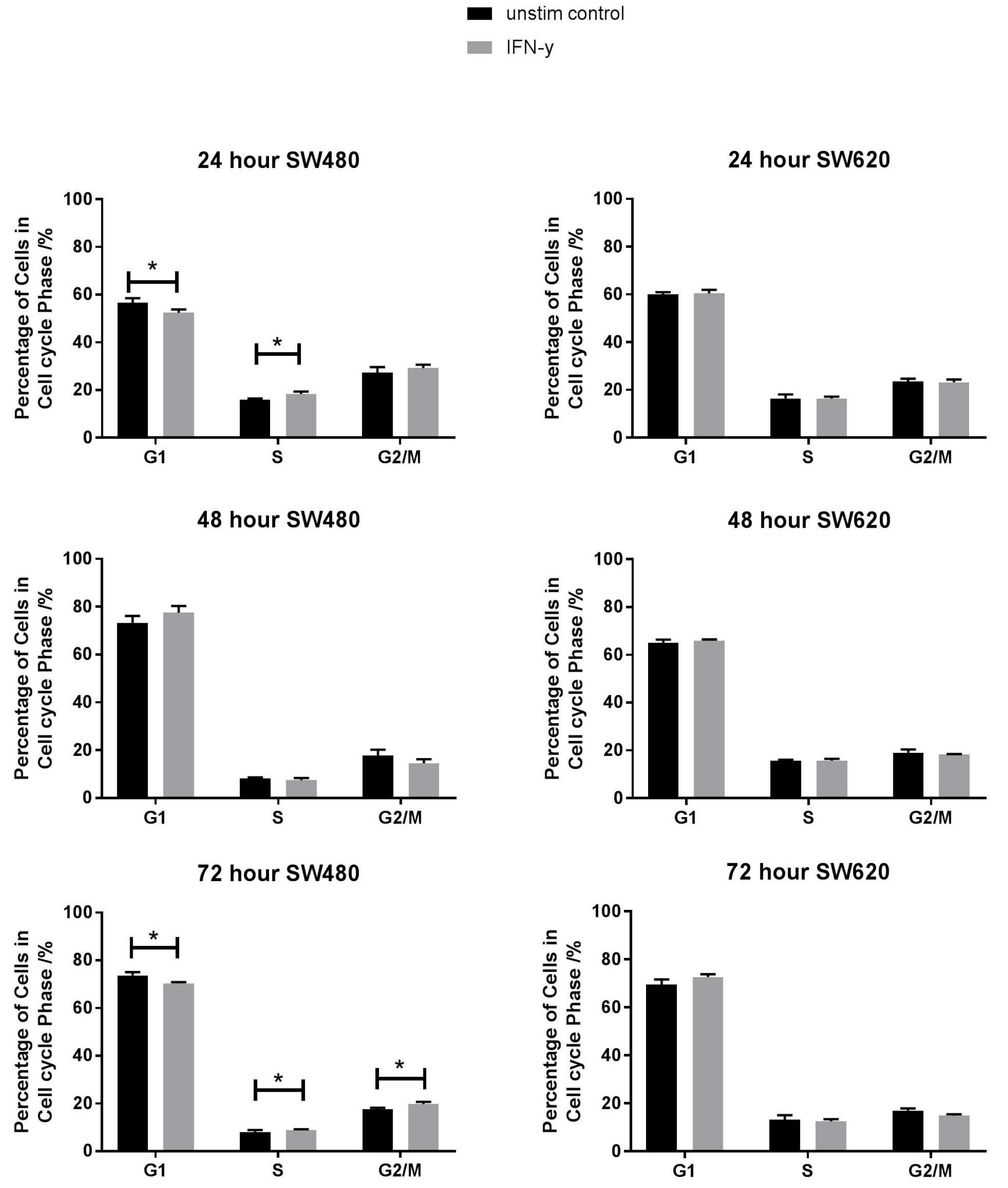


Figure 3: Cell cycle distribution was analysed at 24, 48 and 72 hrs when unstimulated or treated with IFN-γ 20ng/ml. Analysis is representative of triplicate wells, each bar represents mean ± standard deviation, symbol <sup>\*</sup> denotes statistical significance ( $P < 0.05$ ).

IFN-γ stimulation resulted in significant S phase accumulation and G1 reduction at 24hrs in SW480 cells. Additionally, IFN-γ stimulation caused significant S phase and G2/M phase accumulation and G1 reduction at 72 hrs in SW480 cells. However, no significant effects were observed in the SW620 cell line when stimulated with IFN-γ (Figure 3).

Due to these findings, we wanted to investigate if polyphenols could enhance the effect IFN-γ on cell cycle and other cellular processes as they have be shown to affect cycle in previous work.

### The effect of the polyphenols apigenin and quercetin on ATP levels/cell viability

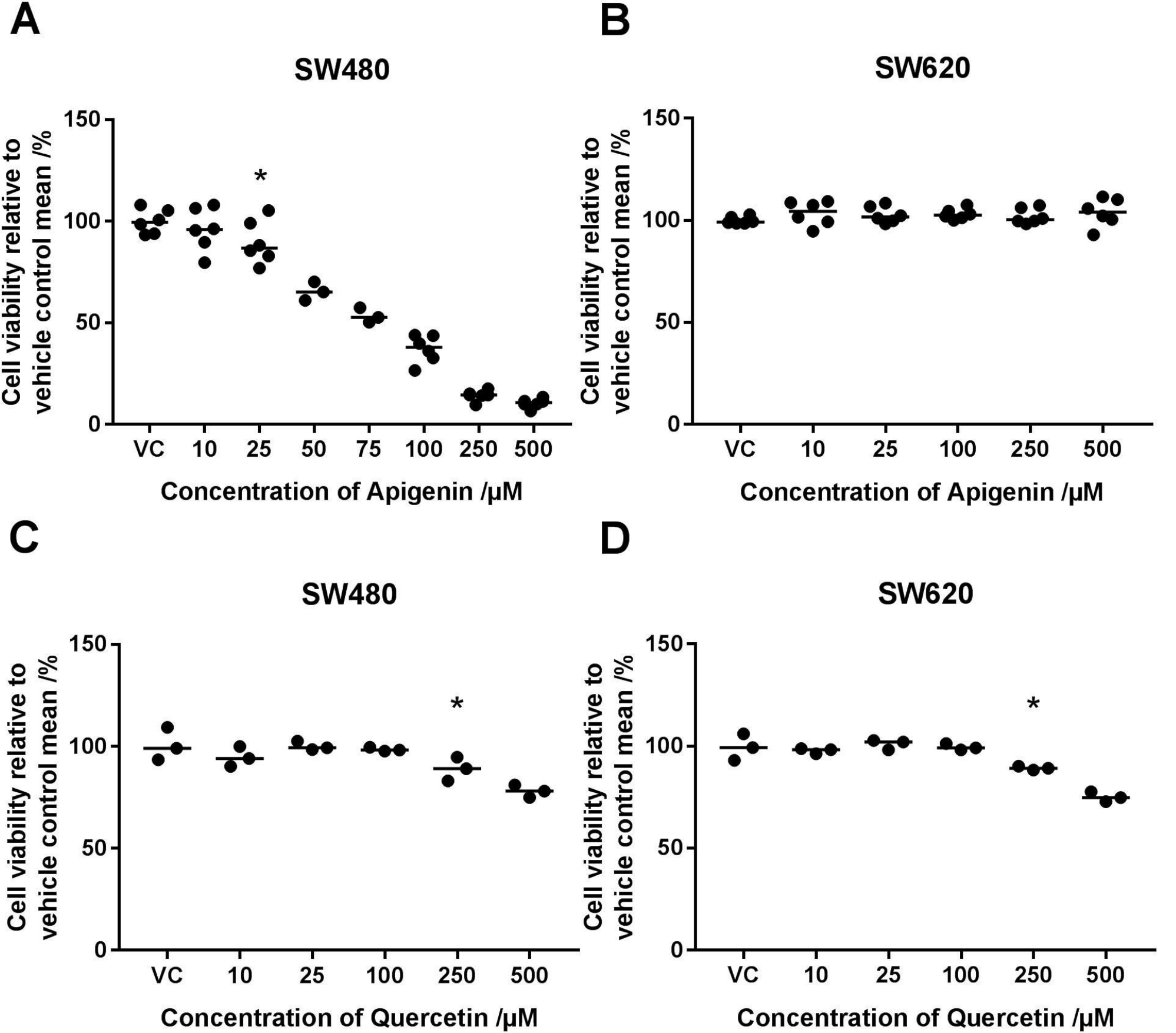


Figure 4: The effect of apigenin and quercetin on the ATP levels as a measure of cell viability in SW480 and SW620 cells after 24 hours; evaluated by cell titre glo assay. Data was normalised to the vehicle control (VC) mean which was assigned 100% cell viability. Data expressed as median and ranges. Statistical significance was determined by comparison with the vehicle control, and statistical significance was set at  $P < 0.05$  which was determined as the lowest significant dose.

Apigenin displayed a lowest significant dose of 25 µM in the primary SW480 CRC cells (Figure 4A) but no effect was seen in the metastatic SW620 CRC cells when treated with a dose up to 500 µM (Figure 4B).

Quercetin displayed a lowest significant dose of 250 µM for both CRC cell lines (Figure 4C & 4B).

Apigenin was chosen for further experimentation as it had the most physiologically relevant lowest significant dose.

### The effect of combination treatment of IFN-γ and apigenin on cell cycle progression

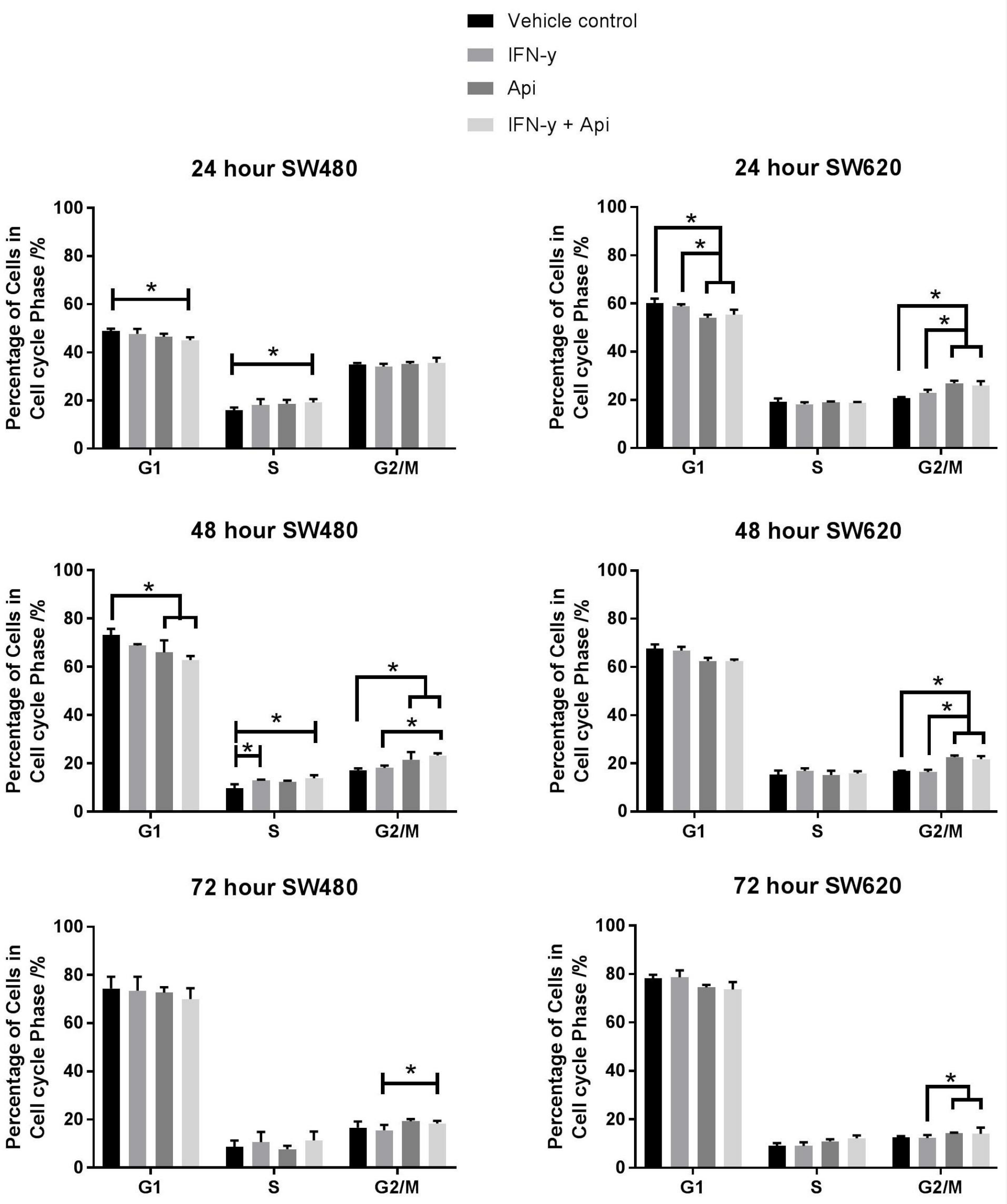


Figure 5: Cell cycle distribution was analysed at 24, 48 and 72 hrs after treatment of vehicle control, IFN-γ 20ng/ml, apigenin (Api) 25 µM or IFN-γ 20ng/ml + apigenin 25 µM (IFN-γ + Api). Analysis is representative of triplicate wells, each bar represents mean ± standard deviation, symbol <sup>\*</sup> denotes statistical significance ( $P < 0.05$ ).

Apigenin alone and in combination caused significant G2/M phase accumulation and G1 phase reduction in the SW620 cell line at 24hrs. The same effect was seen again at 48hrs in both the SW480 and SW620 cell lines (Figure 5).

IFN-γ displayed little to no significant effect on either of the SW480 or SW620 cell lines. Apigenin causes G2/M arrest but does not work synergistically with interferon-gamma.

## Conclusions

We found that IFN-γ 20ng/ml is a sufficient dose to stimulate the increased expression of PD-L1 in both the primary and metastatic human colorectal cancer cell lines. This upregulation of PD-L1 in response to IFN-γ stimulation is a strategy for the CRC cells to evade the immune system. The same dose of IFN-γ however was not significant enough too effect the cell cycle or cell viability of the either SW480 or SW620 cell lines.

The polyphenol apigenin displayed a significantly lower significant dose in the SW480 cell line than quercetin, although had no apparent effect on the metastatic SW620 cell line. However, quercetin did lower ATP production in the SW620 cell line at 250 µM. These data suggest the two polyphenols have potential anti-cancer properties, however, apigenin has shown to be more potent in the primary SW480 cell line. Polyphenols could be considered as effective adjuvants to immunotherapies .

## Future research

With funding from the British society of immunology we are looking to continue researching the combination effect of IFN-γ and apigenin on PD-L1 expression, apoptosis and further cell cycle analysis.

Apigenin has been shown to be an inhibitor of STAT1 and STAT3 (Hui-Hui Cao, *et al* 2016), so we hypothesize that apigenin may be able to reduce the level of PD-L1 induced by IFN-γ.

## References

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